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Research letter

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Synergistic effect of crude oil plus dispersant on bacterial community in a louisiana salt marsh sediment

Mohammed Al-Jawasim, Kewei Yu and Joong-Wook Park*

Department of Biological and Environmental Sciences, Troy University, Troy, AL 36082, USA

*Corresponding author: Department of Biological and Environmental Sciences, Troy University, Troy, AL 36082, USA. Tel: +334-808-6416; Fax: +334-670-3662; E-mail: jwpark@troy.edu

One sentence summary: Synergistic effect of crude oil plus dispersant (Corexit 9500A) significantly altered indigenous bacterial communities in a Louisiana salt marsh sediment after 30 days of incubation.

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ABSTRACT

A combined effect of crude oil plus dispersant (Corexit 9500A) significantly altered indigenous bacterial communities in a Louisiana salt marsh sediment after 30 days of incubation; the crude oil and/or Corexit 9500A treatments triggered shifts in bacterial communities and the shifted bacterial structure by crude oil plus Corexit 9500A was considerably different from those by either crude oil or Corexit 9500A. However, the synergistic effect of crude oil plus Corexit 9500A was not observed after 7 days of incubation; the bacterial community was slightly shifted by Corexit 9500A and the crude oil did not trigger any bacterial community shift after 7 days of incubation. DNA sequencing data indicated that *Chromobacterium* species was enriched in the Corexit 9500A microcosms after 7 days of incubation, while *Pseudomonas, Advenella, Acidocella* and *Dyella* spp. were enriched after 30 days of incubation. *Parvibaculum* was a dominant species in the crude oil microcosms after 30 days of incubation. *Rhodanobacter, Dyella* and *Frateuria* spp. were dominant in crude oil plus Corexit 9500A microcosms after 30 days of incubation. Our data show that the effect of crude oil plus Corexit 9500A on bacterial community is synergistic, and thus the dispersant effect should be considered with the spilled oil to correctly evaluate the environmental impact.

Keywords: crude oil; Corexit; synergistic effect; dispersed oil; bacterial community; salt marsh

INTRODUCTION

Dispersant application is one of the strategies to mitigate oil spill impacts (Fiocco and Lewis 1999) that has been applied since the 1950s (Ramachandran *et al.* 2004). Because of the amphipathic nature of surfactants (Canevari 1969; Fiocco and Lewis 1999), the dispersants arrange themselves at oil–water interfaces to minimize the interfacial tension and break oil slicks into tiny droplets that settle in the water column (Goodbody-Gringley *et al.* 2013; Prince and Butler 2013). Consequently, the dispersant application increases biodegradation of crude oil by enhancing oil bioavailability to a large number of bacterial species, particularly hydrocarbon-degrading bacteria (Fiocco and Lewis 1999; Prince, Lessard and Clark 2003). Varadaraj *et al.* (1995) reported that Corexit 9500A—a dispersant used extensively in the Deepwater Horizon oil spill—positively affected the microbial growth and enhanced the rate of microbial oil degradation, since sorbitan in Corexit 9500A can be served as a nutrient for microbial growth.

On the other hand, a substantial amount of research has demonstrated the dispersant toxicity. Singer *et al.* (1996) showed that Corexit 9500A can elicit acute toxic effects to aquatic organisms such as kelp forest mysid and red abalone. Hamdan and Fulmer (2011) demonstrated that reproduction and viability

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of two oil-degrading bacterial isolates were significantly inhibited by Corexit 9500A. Besides the toxicity of dispersants themselves, toxic effects of dispersed oil or chemically enhanced water-accommodated fraction (CEWAF) has been extensively studied (Ramachandran *et al.* 2004; Place *et al.* 2010). A considerable amount of research supports that CEWAF is toxic to fish (Ramachandran *et al.* 2004, 2006; Gardiner *et al.* 2013; Rico-Martinez, Snell and Shearer 2013; Adams, Sweezey and Hodson 2014; Dussauze *et al.* 2015), copepod (Gardiner *et al.* 2013; Lee *et al.* 2013; Cohen, McCormick and Burkhardt 2014), crab (Chase *et al.* 2013), shrimp/abalone (Rico-Martinez, Snell and Shearer 2013), sea urchin embryo (Rial, Vazquez and Murado 2014), coral larva (Goodbody-Gringley *et al.* 2013), rotifera (Singer *et al.* 1998) and diatom (Hook & Osborn, 2012). However, there still are controversial issues on synergistic toxicity of CEWAF as compared to non-dispersed oil. Some researchers insisted no synergistic toxicity of CEWAF (Adams, Sweezey and Hodson 2014; Dussauze *et al.* 2015), while others claimed synergistic toxic effect of oil and dispersant (Singer *et al.* 1998; Rico-Martinez, Snell and Shearer 2013).

Various eukaryotes were used as model organisms to evaluate the synergistic effect of CEWAF, but study is still necessary to investigate the synergistic effect of CEWAF on microbial community. There are several reports to show the effect of CEWAF on microorganisms (Ortmann *et al.* 2012; Baek, Son and Shim 2013), but they did not examine whether the synergistic effect exists. Considering the crucial role of bacteria in marine food webs (Ortmann *et al.* 2012), it is important to investigate the existence of synergistic effect of dispersed oil on marine bacteria. Our research objective is to investigate the synergistic effect of crude oil plus Corexit 9500A on bacteria in aLouisiana salt marsh sediment. Bacterial community shifts after expose to crude oil and/or Corexit 9500A were monitored after 7 and 30 days of incubation.

MATERIALS AND METHODS

Sampling area

A sediment sample was collected in May 2013 from a salt marsh at the eastern side of Lake Pontchartrain, Louisiana (N30° 08.782′ W89° 44.665′). The site was dominated by two marsh plants, *Spartina alterniflora* and *S. patens*. After eliminating the surface vegetation, the top 30 cm sediments were collected, stored in sterilized containers, transported on ice to the laboratory and stored at 4°C before use.

Chemical analysis

The sediment sample was sent to the Central Analytical Instruments Research Laboratory, Louisiana State University (Baton Rouge, LA) for chemical analysis. EPA methods 200.7, 300.0 and 365.3 were used to analyze metals, anions and total phosphorous, respectively. Chemical analyses of the sediment were performed to assess factors that might affect the indigenous bacterial community (Table S1, Supporting Information).

Microcosm setup and DNA extraction

West Texas Intermediate (WTI), also known as Texas Light Sweet crude oil, was purchased from Texas Raw Crude (Midland, TX) and the dispersant Corexit 9500A was generously provided by the Nalco Energy Services (Sugar Land, TX). Four sets of microcosms were (1) untreated control, (2) microcosms with 0.2% (v/w) of Corexit 9500A, (3) microcosms with 2% (v/w) of WTI and (4) microcosms with 0.2% of Corexit 9500A and 2% of WTI. The microcosms were set up in triplicate. One gram of sediment was aseptically transferred to 2 mL sterile tubes. Corexit 9500A and/or WTI were added via micropipettes and mixed with the sediment using a tip on a micropipette. The microcosm tubes were sealed with a Teflon-coated cap and incubated without shaking at 30°C. Since approximately two-thirds of air-filled headspace was formed in the microcosm tubes, sediments were incubated under aerobic conditions. The microcosms were sampled after 7 and 30 days of incubation. Total DNA was extracted from 0.3 g of sediment using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) and the DNAs were stored at –20°C prior to analysis.

Polymerase chain reaction (PCR)

Nested PCR method was performed to amplify the bacterial 16S rRNA genes. The primers 27F and 1522R (Giovannoni 1991) were used for a first-round PCR to amplify the entire bacterial 16S rRNA genes. The final volume of the PCR was 50 μL containing 10 pmol of primers, 1 μL of template DNA, 0.25 mM of dNTP, 5 μL of 10x PCR buffer and 1 U of Green *Taq* DNA polymerase (GenScript, Piscataway, NJ). PCR was carried out with a DNA thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA) at an initial temperature of 94°C for 5 min, followed by 30 cycles of 94°C for 20 s, 55°C for 45 s and 72°C for 45 s. A final elongation step was 72°C for 7 min. In the second-round PCR, the primer 341F with GC clamp and 534R were used to amplify the V3 region of the bacterial 16S rRNA gene (Muyzer, De Waal and Uitterlinden 1993). Approximately 1 μL of the first-round PCR product was used as a template for a second-round PCR. PCR conditions and constituents are the same as described above. The resulting PCR products were confirmed by an agarose gel electrophoresis.

Denaturing gradient gel electrophoresis (DGGE) and image analysis

The second-round PCR products were separated on an 8% polyacrylamide gel with a 40–60% denaturing gradient of urea in 1.0× TAE buffer by a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). Approximately 45 μL of PCR products were loaded and the electrophoresis was conducted at 40 V for 15 h. After electrophoresis, gels were stained with ethidium bromide and photographed on a UV transilluminator (Fisher Scientific, Pittsburgh, PA). DGGE images were analyzed by the PyElph software (version 1.4) to construct phylogenetic trees based on DGGE profiles using the unweighted pair group method with arithmetic mean (UPGMA) algorithm.

DNA sequencing and data analysis

The selected DGGE bands were excised using a sterilized blade and incubated with 50 μL of distilled water at 4°C overnight. PCR was carried out to reamplify the eluted DNA with the PCR conditions as described above except that the PCR reaction was performed for 35 cycles. The DGGE analysis was conducted to verify the purity and position of the re-amplified DNAs. If necessary, the DGGE bands were reexcised and the process of PCR-DGGE was repeated until a single DGGE band was confirmed. The resulting PCR products were purified with the MEGAquick-spin Total Kit (iNtRON Biotechnology Inc., Korea) for DNA sequencing. The DNA sequencing was outsourced to the Genewiz Inc. (Genewiz Inc., South Plainfield, NJ). Each sample was sequenced with forward and reverse primers separately. The DNA sequences were assessed using the Chromas Lite Ver. 2.1.1

(Mullen *et al.* 2011), and the basic local alignment search tool (BLAST) algorithm was used to search homologous sequences in the GenBank DNA libraries (Altschul *et al.* 1990).

RESULTS AND DISCUSSION

Effect of crude oil and/or Corexit 9500A after 7 days of incubation

In terms of patterns and intensity of DGGE bands, control samples were almost the same as crude oil treatments (Fig. 1A). The same result was again confirmed by the PyElph analysis (Fig. 1B), which strongly suggests that crude oil treatments have no effect on the bacterial community after 7 days of incubation. Consistent with our observations, it is reported that bacterial abundance and metabolic activity in microcosms were not significantly affected by diesel containing 0.55–55 ppm (dry weight) of polycyclic aromatic hydrocarbons (PAHs) after 7 days of exposure at any concentration tested (Carman, Means and Pomarico 1996). Microbial communities need an adaptation period after they are exposed to a new substrate, which may explain no effect of crude oil on the bacterial community.

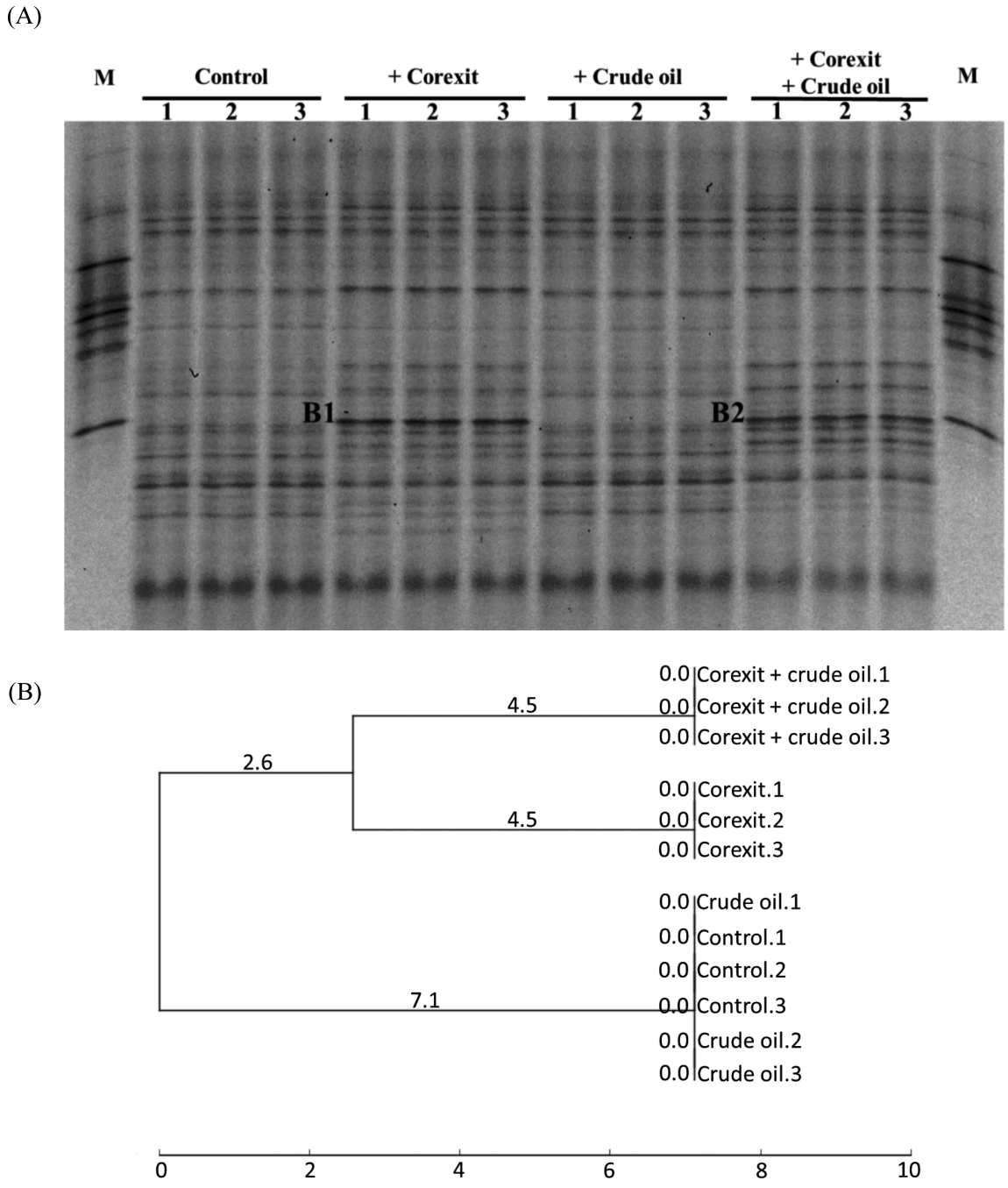

Figure 1. Bacterial community shift after 7 days of incubation. **(A)** DGGE profiles of bacterial 16S rRNA gene fragments in sediment microcosms with 0.2% of Corexit 9500A, 2% of crude oil and 0.2% of Corexit 9500A plus 2% of crude oil. **(B)** UPGMA dendrogram of DGGE profiles. The values on the horizontal lines stand for genetic distances among treatments in percentages. M: custom marker; numbers 1, 2 and 3 represent individual triplicates.

Table 1. Close relatives of major DGGE bands.

Band	Close relatives in GenBank databases	GenBank Accession no	% Identity
B1	*Chromobacterium violaceum* strain ATCC 12472^T	NR_074222	92.74
B2	*Chromobacterium violaceum* strain ATCC 12472^T	NR_074222	92.41
B3	*Pseudomonas* sp. CZ5	GQ903480	91.19
B4	*Advenella kashmirensis* strain W13003	KF147541	76.38
B5	*Acidocella* sp. PFBC	KC590088	98.51
B6	*Dyella ginsengisoli* strain LA-4	EF191354	96.86
B7	*Dyella ginsengisoli* strain LA-4	EF191354	99.37
B10	*Parvibaculum lavamentivorans* strain DS-1^T	NR_074262	97.01
B11	*Rhodanobacter denitrificans* strain 2APBS1^T	NR_102497	97.48
B12	*Dyella ginsengisoli* strain Gsoil 3046^T	NR_041370	99.37
B13	*Frateuria* sp. WJ64	AY495957	99.37

The DGGE profiles of Corexit 9500A treatments were considerably similar to those of crude oil plus Corexit 9500A treatments (Fig. 1A), which was confirmed by the PyElph analysis (Fig. 1B). These data provide additional support that crude oil treatments have no effect on the bacterial community after 7 days of incubation, since the patterns of crude oil plus Corexit 9500A treatments were affected mainly by the Corexit 9500A. It is also noticeable that the effect of Corexit 9500A on bacterial community was detectable after 7 days of incubation. One major DGGE band at the same position and intensity was observed in Corexit 9500A and crude oil plus Corexit 9500A treatments (B1 and B2, Fig. 1A). DNA sequencing data showed these two DGGE bands were homologous to *Chromobacterium violaceum* strain ATCC 12472^T (Table 1). Itah and Essien (2005) demonstrated that *C. violaceum* can extensively degrade hydrocarbons. Since band B1 was appeared in microcosms treated only with Corexit 9500A, it is considered that band B1 was appeared in response to Corexit 9500A. Considering Corexit 9500A is a mixture of hydrocarbons, glycols and sodium dioctyl sulfosuccinate (Bælum *et al.* 2012), our observation suggests that *C. violaceum* thrive by metabolizing hydrocarbons of Corexit 9500A.

Synergistic effect of crude oil plus Corexit 9500A after 30 days of incubation

The bacterial community structures were greatly shifted after 30 days of exposure to Corexit 9500A, crude oil or both (Fig. 2). The UPGMA dendrogram demonstrated that crude oil plus Corexit 9500A treatments were the most phylogenetically distinctive cluster (Fig. 2B). Crude oil plus Corexit 9500A treatments had unique DGGE bands patterns and intensity, which were completely different from those of other treatments or control (Fig. 2A). Hence, our data strongly suggest that crude oil plus Corexit 9500A treatments that contain CEWAF synergistically triggers bacterial community shift. It is also noticeable that this synergistic effect was not observed after 7 days of incubation (Fig. 1). The DNA sequence-based analysis demonstrated that bacterial species thrived in crude oil plus Corexit 9500A treatments were phylogenetically different from those in other treatments (Table 1), which also support the presence of synergistic effect of crude oil plus Corexit 9500A.

Phylogenetic analysis of major DGGE bands after 30 days of incubation

DNA sequence analysis showed that the close relatives of major DGGE bands were associated with biodegradation of crude oil, excluding band B11 which was phylogenetically close to a denitrifying bacterium (Table 1).

The major DGGE bands in Corexit 9500A treatments were bands B3, B4, B5, B6 and B7. The close relative of band B3 was *Pseudomonas* sp. CZ5, a PAH-degrading bacterium isolated from PAH-contaminated sludge samples from a chemical plant in China (Zhou *et al.* 2013). The close relative of band B4 was hydrocarbon-degrading *Advenella kashmirensis* strain W13003 isolated from PAH-contaminated marine sediments in China (Wang *et al.* 2014). The close relative of band B5 was *Acidocella* sp. PFBC, an acidophilic heterotrophic bacterium that can metabolize several aromatic hydrocarbons, such as phenol, benzyl alcohol and benzoate (Jones, Hedrich and Johnson 2013). The close relative of band B6 and B7 was *Dyella ginsengisoli* strain LA-4, which was isolated from an activated sludge of a petrochemical company in China, which can use hydrocarbons as its carbon and energy sources (Li *et al.* 2009a,b). It is considered that the relatives of above oil-degrading bacteria were found in our microcosms, since the microcosms were setup with the estuarine sediment and incubated under aerobic condition with Corexit 9500A, which contains hydrocarbons (Bælum *et al.* 2012).

The bands B8, B9 and B10 were present in microcosms treated with crude oil (Fig. 2A). We failed to sequence the bands B8 and B9, but the band B10 was sequenced. Sequence analysis demonstrated that the band B10 was homologous to *Parvibaculum lavamentivorans* strain DS-1^T (Table 1). This strain was isolated from an activated sludge for its ability to degrade linear alkylbenzene sulfonate surfactants (Schleheck *et al.* 2004, 2007). The genus *Parvibaculum* includes hydrocarbon-degrading species, which were detected in several hydrocarbon-contaminated environments (Alonso-Gutiérrez *et al.* 2009; Paixão *et al.* 2010; Wang *et al.* 2010). Consequently, the enrichment of genus *Parvibaculum* suggests that the hydrocarbon-degrading and/or biosurfactants-producing bacteria may exist in the microcosms treated with crude oil.

The bands B11, B12 and B13 in crude oil plus Corexit 9500A treatments were chosen for DNA sequencing. These bands were phylogenetically homologous to *Rhodanobacter denitrificans* strain 2APBS1^T, *D. ginsengisoli* strain Gsoil 3046^T and *Frateuria* sp. WJ64, respectively (Table 1). *Rhodanobacter denitrificans* strain 2APBS1^T contains nitrate, nitrite, nitric oxide and nitrous oxide reductase genes and is able to perform complete denitrification under anaerobic condition (Green *et al.* 2010; Prakash *et al.* 2012). A recent study showed the inhibition effect of Corexit 9500A on denitrification rate after 2 weeks of incubation (Pietroski, White and DeLaune 2015). The other study reported that Tween 80—a major component of Corexit 9500A (Varadaraj *et al.* 1995)—delayed the

denitrification after 3 weeks of incubation, but the delaying effect of denitrification was not observed and the biodegradation of petroleum hydrocarbons was increased after 6 weeks of incubation (Zhang, Zheng and Lo 2015). Our data suggest the presence of denitrifying bacteria as one of the major species after 30 days of crude oil plus Corexit 9500A treatments (band B11, Fig 2A), which is consistent with previous studies. However, further studies are necessary to confirm this idea. The species *D. ginsengisoli* includes many hydrocarbon-degrading strains such as LA-4 (Li *et al.* 2009a) and MS2 (Chang, Chang and Yuan 2008). It is interesting to note that the bands B6, B7 and B12 were appeared at different positions, but they were homologous to the same species *D. ginsengisoli* (Table 1). The genus *Frateuria* includes several bacterial species such as *Frateuria* sp. ANA-18 (Murakami *et al.* 2003) and *Frateuria aurantia* (Zemb *et al.* 2012) that can metabolize hydrocarbons as the sole source of carbon and energy.

CONCLUSION

While a 7-day exposure did not show the synergistic effect of crude oil plus Corexit 9500A, the synergistic effect was observed

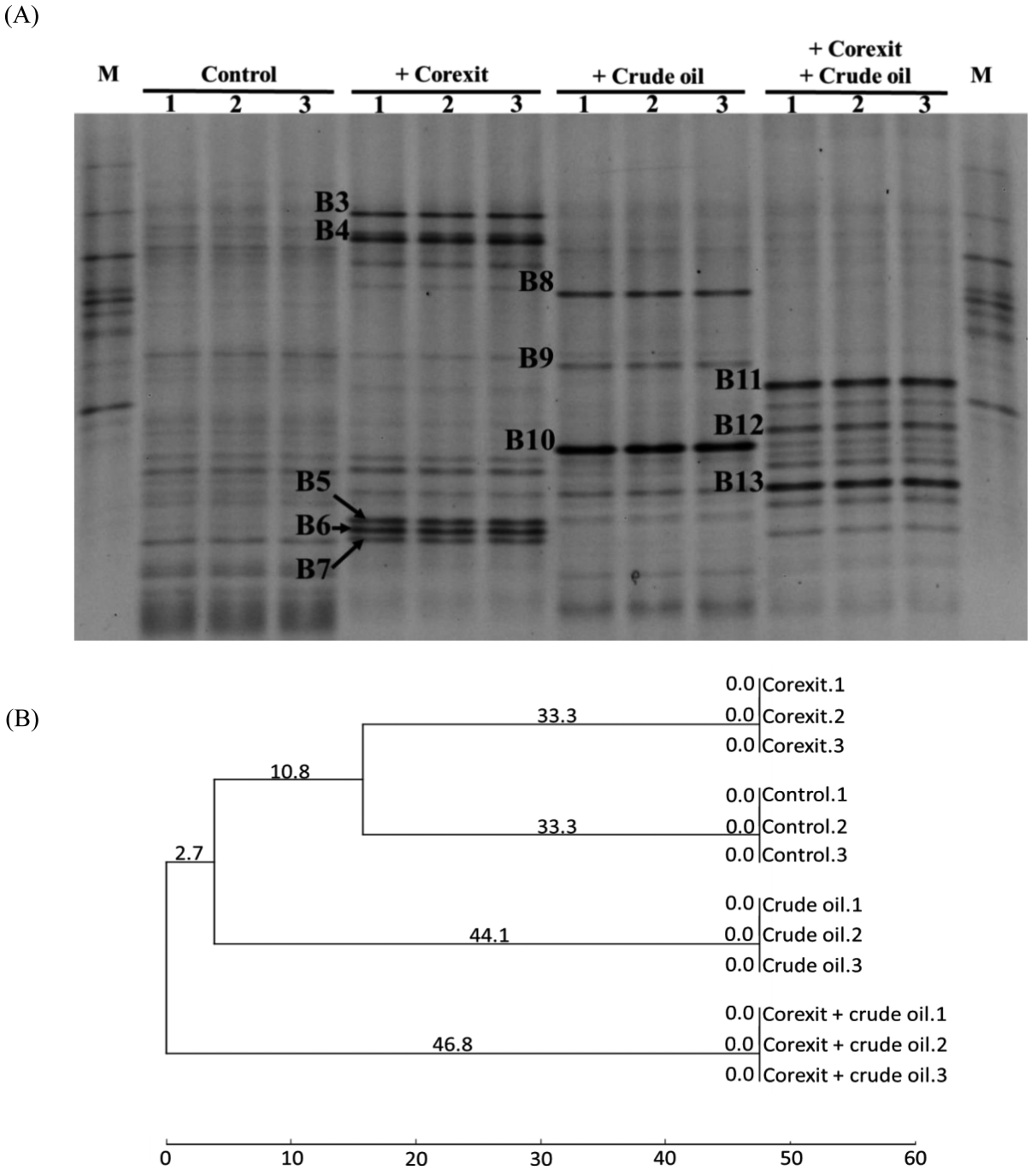

Figure 2. Bacterial community shift after 30 days of incubation. **(A)** DGGE profiles of bacterial 16S rRNA gene fragments in sediment microcosms with 0.2% of Corexit 9500A, 2% of crude oil and 0.2% of Corexit 9500A plus 2% of crude oil. **(B)** UPGMA dendrogram of DGGE profiles. The values on the horizontal lines stand for genetic distances among treatments in percentages. M: custom marker; numbers 1, 2 and 3 represent individual triplicates.

after a 30-day exposure. Considering crude oil plus Corexit 9500A contain CEWAF, our data support the presence of synergistic effect of CEWAF on indigenous bacteria in the Louisiana salt marsh sediment. Our findings strongly suggest that the dispersant effect should be considered with the spilled oil to correctly evaluate the environmental impact.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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Conflict of interest. None declared.

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